

# Imaging Reversible Mitochondrial Membrane Potential Dynamics with a Masked Rhodamine Voltage Reporter

Pavel E. Z. Klier, Julia G. Martin, and Evan W. Miller\*



Cite This: *J. Am. Chem. Soc.* 2021, 143, 4095–4099



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**ABSTRACT:** Mitochondria are the site of aerobic respiration, producing ATP via oxidative phosphorylation as protons flow down their electrochemical gradient through ATP synthase. This negative membrane potential across the inner mitochondrial membrane ( $\Delta\Psi_m$ ) represents a fundamental biophysical parameter central to cellular life. Traditional, electrode-based methods for recording membrane potential are impossible to implement on mitochondria within intact cells. Fluorescent  $\Delta\Psi_m$  indicators based on cationic, lipophilic dyes are a common alternative, but these indicators are complicated by concentration-dependent artifacts and the requirement to maintain dye in the extracellular solution to visualize reversible  $\Delta\Psi_m$  dynamics. Here, we report the first example of a fluorescent  $\Delta\Psi_m$  reporter that does not rely on  $\Delta\Psi_m$ -dependent accumulation. We redirected the localization of a photoinduced electron transfer (PeT)-based indicator, Rhodamine Voltage Reporter (RhoVR), to mitochondria by masking the carboxylate of RhoVR 1 as an acetoxymethyl (AM) ester. Once within mitochondria, esterases remove the AM ester, trapping RhoVR inside of the mitochondrial matrix, where it can incorporate within the inner membrane and reversibly report on changes in  $\Delta\Psi_m$ . We show that this Small molecule, Permeable, Internally Redistributing for Inner membrane Targeting Rhodamine Voltage Reporter, or SPIRIT RhoVR, localizes to mitochondria across a number of different cell lines and responds reversibly to changes in  $\Delta\Psi_m$  induced by exceptionally low concentrations of the uncoupler FCCP without the need for exogenous pools of dye (unlike traditional, accumulation-based rhodamine esters). SPIRIT RhoVR is compatible with multi-color imaging, enabling simultaneous, real-time observation of cytosolic  $\text{Ca}^{2+}$ , plasma membrane potential, and reversible  $\Delta\Psi_m$  dynamics.

Membrane-bound organelles maintain gradients of ions across their membranes, resulting in a membrane potential ( $V_{\text{mem}}$ ), or voltage, difference relative to the cytosol.<sup>1</sup> Mitochondria are perhaps the most well-known example of this phenomenon: as the locus of oxidative phosphorylation,<sup>2</sup> the chemical potential energy in cellular fuel sources is used to pump  $\text{H}^+$  across the mitochondrial inner membrane and into the intermembrane space. In normally respiring mitochondria, this results in a voltage gradient of approximately 160 mV, with the matrix negative relative to the intermembrane space. The magnitude of this mitochondrial electrochemical potential ( $\Delta\Psi_m$ ) profoundly influences the rate of ATP synthesis,<sup>3</sup> is linked to calcium release,<sup>4</sup> and is often misregulated in cancer metabolism.<sup>5</sup>

Traditionally,  $V_{\text{mem}}$  is measured using patch-clamp electrophysiology, but the invasive nature of this technique, the small size of mitochondria, and the sequestration of the mitochondrial inner membrane within both the plasma membrane and the outer mitochondrial membrane make electrode-based methods impossible to implement for mitochondria inside of living cells. Optical methods are a promising route for studying voltage in membrane-bound organelles because a membrane-permeable dye can be used in intact cells to provide a minimally invasive readout of the membrane potential of an organelle in its physiological niche.<sup>6</sup>

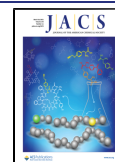
The vast majority of reported fluorescent sensors of  $\Delta\Psi_m$  involve a lipophilic, cationic fluorophore that accumulates in mitochondria in proportion to the negative  $\Delta\Psi_m$ . The change in voltage is then read out by measuring the intensity of

mitochondrial fluorescence,<sup>7</sup> the change in color as the dye forms spectrally shifted aggregates,<sup>8,9</sup> the FRET efficiency between a static donor and a mobile acceptor,<sup>10,11</sup> or the physical partition of the dye between mitochondria and another subcellular compartment.<sup>12,13</sup> A commonly used class of dyes is rhodamine esters (Scheme S1), which report changes in  $\Delta\Psi_m$  via the intensity of mitochondrial fluorescence. However, this approach has some technical limitations. First, at high concentrations, rhodamines and other lipophilic dyes will self-quench, confounding interpretation of intensity changes.<sup>14</sup> Second, dye must be continuously present in the bath solution to compensate for diffusion out of the mitochondria that occurs when no dye is present extracellularly. It is impossible to leave the dye solution on the sample in some experimental configurations. Finally, because all of the mechanisms listed above rely on diffusion, rapid voltage changes on time scales of milliseconds will not be observed.<sup>15</sup>

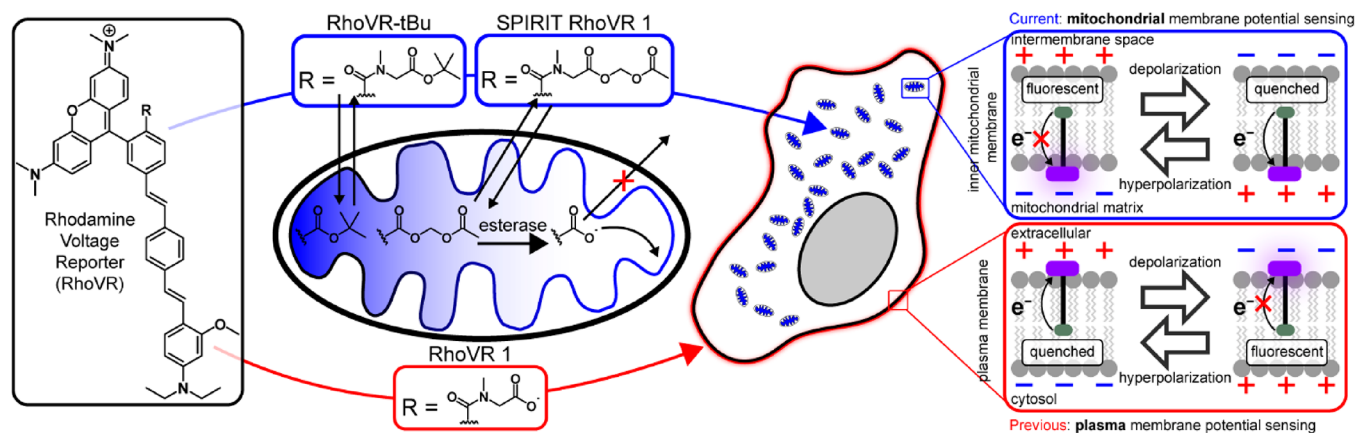
Voltage-sensitive fluorophores that sense changes in  $V_{\text{mem}}$  via a photoinduced electron transfer (PeT) mechanism<sup>16</sup> can address some of the limitations of diffusion-based rhodamine esters. The lipophilic molecular wire of voltage-sensitive

Received: December 18, 2020

Published: March 12, 2021



## Scheme 1. Imaging Mitochondrial Membrane Potential Dynamics with Permeable SPIRIT RhoVR Indicators

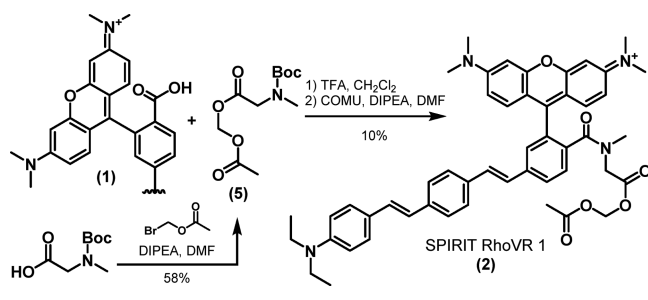


fluorescent (VF) dyes drives membrane localization, and anionic carboxylate or sulfonate groups prevent internalization. In the course of developing rhodamine-based voltage reporters (RhoVR dyes), we found that the *t*Bu ester of RhoVR (Scheme 1) localized to internal structures, whereas the free carboxylate (RhoVR 1, Scheme 1) localized to plasma membranes.<sup>17</sup>

We hypothesized that RhoVR-*t*Bu localized to mitochondria, on account of its overall cationic and lipophilic nature, and would show  $\Delta\Psi_m$ -dependent accumulation in these structures, similar to classic rhodamine esters. We wondered whether a labile ester, like acetoxymethyl (AM) ester, would allow RhoVR to accumulate in the mitochondrial matrix, where esterases<sup>18</sup> could hydrolyze the ester, trapping RhoVR 1 and enabling it to insert to the inner membrane. The revealed carboxylate would prevent it from acting like an “accumulation”-type rhodamine ester, and it would then sense voltage via a PeT-based mechanism. This Small molecule, Permeable, Internally Redistributing for Inner membrane Targeting RhoVR, or SPIRIT RhoVR 1, represents the first of a new class of  $\Delta\Psi_m$  indicators that does not rely on molecular accumulation for voltage sensing.

SPIRIT RhoVR1 is synthesized in three steps (Scheme 2). Esterification of *N*-*tert*-butyloxycarbonyl sarcosine (Boc-sarc,

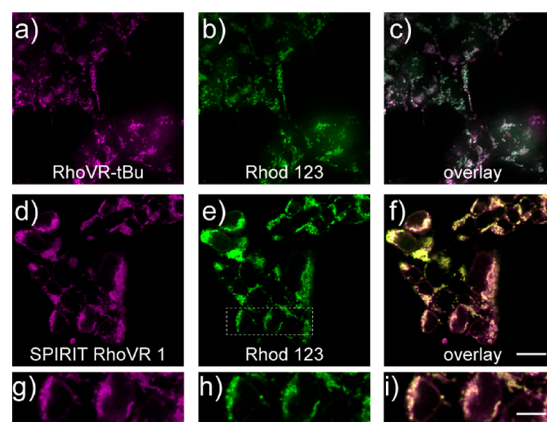
## Scheme 2. Synthesis of SPIRIT RhoVR



5) with bromomethyl acetate provides the AM ester of 5 in 58% yield. Removal of the *N*-Boc protecting group with trifluoroacetic acid (TFA), followed immediately by peptide bond formation with carboxy-RhoVR (1) mediated by HATU, produces SPIRIT RhoVR 1 (2) in 10% yield. A control compound, SPIRIT RhoVR 0 (4), is synthesized via a similar route from RhoVR 0 carboxylate 3 (Scheme S4). RhoVR 0 derivatives lack the aniline group required for voltage sensitivity. RhoVR 0, like RhoVR 1, localizes to the plasma

membrane but is not voltage-sensitive.<sup>19</sup> SPIRIT RhoVR 0 provides an important negative control for characterization of mitochondrial voltage responses in cells. The new SPIRIT RhoVR derivatives possess emission and excitation profiles nearly identical to those of the parent RhoVR compounds (Figure S1, Table S1).

Both SPIRIT RhoVR 1 and RhoVR-*t*Bu localize to mitochondria. Confocal imaging of HEK 293T cells stained with SPIRIT RhoVR 1 (Figure 1a–c) or RhoVR-*t*Bu (Figure

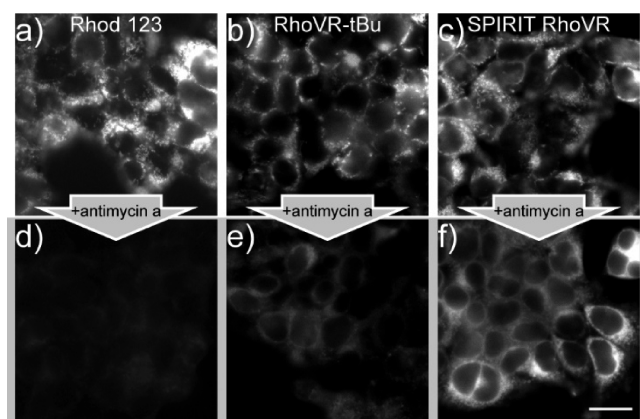


**Figure 1.** SPIRIT RhoVR 1 localizes to mitochondria in mammalian cells. Confocal fluorescence microscopy images of HEK cells stained with either (a) RhoVR-*t*Bu (250 nM), (d) SPIRIT RhoVR 1 (250 nM), or (b, e) rhodamine 123 (250 nM). Overlay images of rhodamine 123 and either (c) RhoVR-*t*Bu or (f) SPIRIT RhoVR 1. (g–i) Expanded views of the boxed region in panel e. Scale bar is 20  $\mu\text{m}$  (a–f) or 10  $\mu\text{m}$  (g–i).

1d–f) display punctate, intracellular staining that co-localizes with mitochondria-targeted rhodamine 123 (Movie S1) and possess near-unity Pearson correlation (Figure S2a). In contrast, RhoVR 1, which senses plasma membrane potential, does not co-localize with rhodamine 123 (Figure S2b–d) and has a low Pearson correlation coefficient of  $0.28 \pm 0.04$  (Figure S2a). Localization of SPIRIT RhoVR 1 and 0 in mitochondria is generalizable across a range of cell lines (Figure S2e,f). SPIRIT RhoVR 1 shows very little co-localization with lysosomes (Figure S3).

Unlike traditional rhodamine esters, SPIRIT RhoVR 1 remains localized to mitochondria following depolarization. Mitochondrial depolarization causes accumulation-based dyes

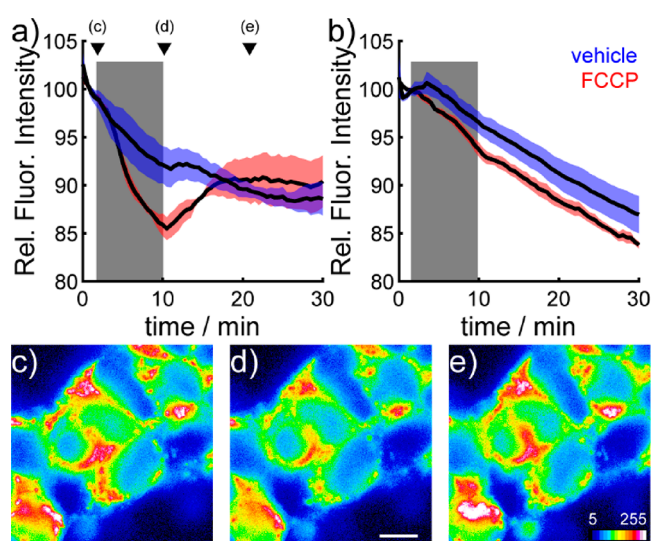
like rhodamine 123 to diffuse out of mitochondria,<sup>20</sup> whereas SPIRIT RhoVR1 should be retained in mitochondria, since loss of AM ester reveals a charged carboxylate which prevents RhoVR-type molecules from crossing cellular membranes (Scheme 1).<sup>17</sup> In contrast, intact esters of RhoVR-type molecules should accumulate in hyperpolarized mitochondria and diffuse out of depolarized mitochondria. We loaded HEK 293T cells with rhodamine esters rhodamine 123, RhoVR-tBu, and SPIRIT RhoVR 1 and induced mitochondrial membrane depolarization (Figure 2) with antimycin A<sup>21</sup> (Figure S4).



**Figure 2.** SPIRIT RhoVR1 is retained in mitochondria after dissipation of  $\Delta\Psi_m$  with antimycin A. Wide-field fluorescence microscopy of rhodamine or RhoVR derivative (250 nM) in the (a–c) absence or (d–f) presence of antimycin A (5  $\mu\text{g}/\text{mL}$ ). Wide-field fluorescence microscopy of SPIRIT RhoVR1 in the (c) absence or (d) presence of 5  $\mu\text{g}/\text{mL}$  antimycin A for 90 min. Scale bar is 20  $\mu\text{m}$ .

Antimycin A inhibits complex III, preventing electron transfer into cytochrome *c* and abolishing  $\text{H}^+$  transfer from the matrix to the inner membrane space, resulting in a loss of  $\Delta\Psi_m$ . Rhodamine 123 and RhoVR-tBu exhibit significant ( $p = 0.04$  and  $0.02$ , Figure S4e) washout from mitochondria and dilution into the dye-free external solution upon antimycin A treatment (Figure 2a/d and b/e), displaying fluorescence approximately 50% and 60% as bright as that of cells treated with vehicle (Figure S4e). By comparison, SPIRIT RhoVR 1 fluorescence is retained (Figure 2c/f) at levels nearly identical to that of vehicle controls (Figure S4e). SPIRIT RhoVR 1 remains localized to mitochondria, even after antimycin A-induced depolarization (Figure S4f–h).

SPIRIT RhoVR 1 tracks depolarizations and hyperpolarizations in  $\Delta\Psi_m$  with high fidelity. HEK 293T cells loaded with SPIRIT RhoVR 1 and treated with the protonophore carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone<sup>22</sup> (FCCP, 500 nM) to collapse the  $\Delta\Psi_m$  show a decrease in fluorescence (Figure 3a, red), consistent with a depolarization of the inner mitochondrial membrane, which results in an increased rate of PeT and a subsequent decrease in fluorescence intensity (Scheme 1). Fluorescence intensity is restored to baseline levels upon washout of FCCP (Figure 3a,c–e, Movie S2). Cells loaded with SPIRIT RhoVR 1 and treated identically, but with a vehicle control (EtOH), show no change in fluorescence intensity (Figure 3a, blue). Identical experiments using cells loaded with SPIRIT RhoVR 0 (which lacks an aniline and is therefore not voltage-sensitive via a PeT-based mechanism)<sup>17</sup> show no change in fluorescence (Figure 3b, Movie S3),



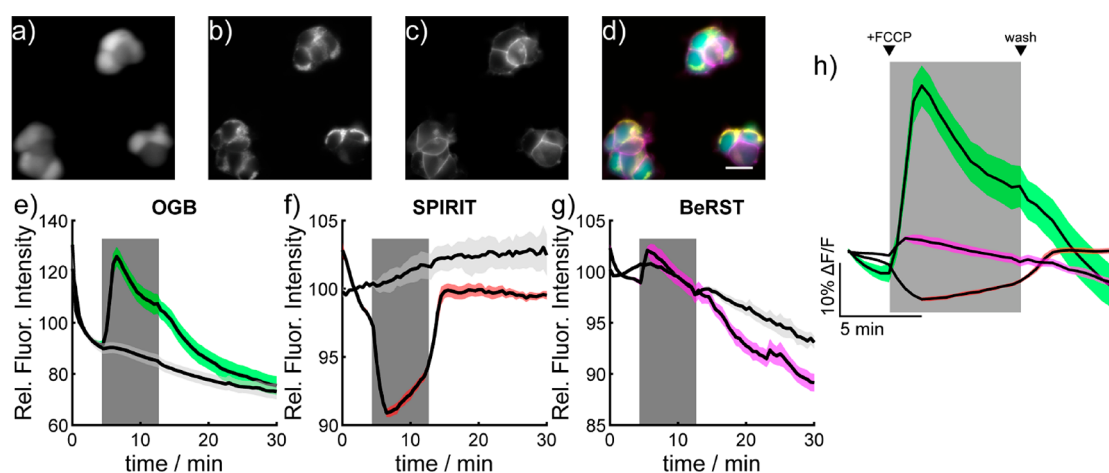
**Figure 3.** SPIRIT RhoVR 1 reports on  $\Delta\Psi_m$  dynamics in HEK cells. Plot of fluorescence intensity vs time for HEK cells stained with (a) SPIRIT RhoVR 1 (150 nM) or (b) SPIRIT RhoVR 0 (150 nM). At 2 min into the experiment (beginning of gray box), cells were perfused with either vehicle (ethanol, blue) or FCCP (500 nM, red). At 10 min (end of gray box), cells were perfused with HBSS. Data are mean (black line)  $\pm$  SEM (colored shading) for three separate experiments. Representative pseudocolor images of SPIRIT RhoVR 1-loaded HEK cells (c) before, (d) during, and (e) after treatment with FCCP (500 nM). Scale bar is 10  $\mu\text{m}$  for all images. Arrowheads in panel a indicate the time points of the representative images in panels c–e.

consistent with a PeT mechanism of voltage sensing. The slow decay of fluorescence, even in vehicle controls, is likely due to a combination of photobleaching and slow leakage of unhydrolyzed SPIRIT RhoVR. TMRM, a traditional, accumulation-based  $\Delta\Psi_m$  indicator, cannot report on reversible changes under these conditions (Figure S5). Higher concentrations of FCCP (>500 nM) should not be used. The micromolar levels of FCCP reported in the literature<sup>14,23</sup> may cause artifacts in dyes that should not have a voltage response (Figure S6). SPIRIT RhoVR 1 can track reversible changes in  $\Delta\Psi_m$  across several common cell lines, including COS7, MCF-7, and HeLa (Figure S7).

SPIRIT RhoVR 1 responds reversibly to changes in  $\Delta\Psi_m$ , allowing the real-time observation of voltage dynamics without the need for an extracellular pool of indicators, and can be used to detect  $\Delta\Psi_m$  changes induced by nanomolar concentrations of FCCP. Because of its robust response to  $\Delta\Psi_m$  dynamics, we thought that SPIRIT RhoVR 1 could be an important component of methods to monitor multiple cellular physiological parameters simultaneously. We stained HEK 293T cells with Oregon Green BAPTA (OGB, to monitor intracellular  $\text{Ca}^{2+}$ ),<sup>24</sup> BeRST 1, a far-red plasma  $V_{\text{mem}}$  indicator,<sup>25</sup> and SPIRIT RhoVR 1. Cells loaded in this way show clear cytosolic fluorescence in the OGB channel (Figure 4a), mitochondrial-localized tetramethyl rhodamine fluorescence from SPIRIT RhoVR 1 (Figure 4b), and plasma membrane-localized fluorescence in the far-red channel from BeRST 1 (Figure 4c). Importantly, we are able to isolate the excitation and emission spectra of each indicator, minimizing excitation and emission cross-talk (Figure S8) to allow real-time, three-color imaging (Figure 4d, Movie S4).

FCCP perfusion of cells with the three indicators gives an increase in OGB fluorescence with a peak change of nearly





**Figure 4.** Simultaneous, multi-color imaging of mitochondrial membrane potential, cytosolic  $\text{Ca}^{2+}$ , and plasma membrane potential in mammalian cells. Wide-field epifluorescence images of HEK cells stained with (a) OGB (500 nM), (b) SPIRIT RhoVR 1 (150 nM), and (c) BeRST 1 (50 nM). (d) An overlay of the images showing cytosolic localization of OGB (green), mitochondrial localization of SPIRIT RhoVR 1 (yellow), and plasma membrane localization of BeRST (magenta). Scale bar is 20  $\mu\text{m}$ . Plots of fluorescence vs time from HEK cells stained with (e) OGB, (f) SPIRIT RhoVR 1, or (g) BeRST. At 4 min into the experiment (beginning of gray box), cells were perfused with either vehicle (ethanol, light gray) or FCCP (500 nM, colored trace). At 4 min (end of gray box), cells were recovered by perfusion with HBSS. Data are mean (black line)  $\pm$  SEM (colored shading) for three separate experiments. (h) Zoomed-in plot of the response of OGB (green), SPIRIT RhoVR 1 (red), and BeRST 1 (magenta). The gray box indicates the start and end of FCCP perfusion.

30% over baseline, followed by a decay to steady-state levels of  $\sim 20\%$  before returning to baseline upon removal of FCCP (Figure 4e), consistent with reports of  $\text{Ca}^{2+}$  release from mitochondrial stores in response to FCCP.<sup>26</sup> Perfusion of vehicle control gives no change in OGB fluorescence (Figure 4e, light gray). The increase in cytosolic  $\text{Ca}^{2+}$  occurs simultaneously with the depolarization of  $\Delta\Psi_m$ , as measured by SPIRIT RhoVR 1 (Figure 4f,h). At the same time, BeRST fluorescence indicates a small FCCP-induced depolarization at the plasma membrane (Figure 4g, magenta).<sup>27,28</sup> Analysis of individual cell responses allows examination of the similarity of responses across all imaged cells (Figure S9).

In summary, we present the design, synthesis, and application of SPIRIT RhoVR 1, the first in its class of voltage-sensitive fluorophores that report on changes to  $\Delta\Psi_m$  via a PeT-based mechanism. SPIRIT RhoVR 1 shows excellent localization to mitochondria, is retained within the mitochondria even after the dissipation of  $\Delta\Psi_m$ , and can reversibly respond to both hyper- and depolarization of  $\Delta\Psi_m$  without the need for exogenous pools of dye in solution. In the future, we envision a palette of mitochondrial-targeted indicators, pairing with non-native enzymes to achieve enhanced targeting to mitochondria and other organelles, and development of indicators with improved brightness for monitoring rapid organelle membrane potential changes.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.0c13110>.

Synthetic details; supporting Schemes S1–S4, Table S1, and Figures S1–S9; and additional data (PDF)

Movie S1, mitochondrial localization of SPIRIT RhoVR1 in HEK cells (MP4)

Movie S2, SPIRIT RhoVR1 senses FCCP-induced mitochondrial depolarizations (AVI)

Movie S3, SPIRIT RhoVR0 is insensitive to FCCP-induced mitochondrial depolarizations (AVI)

Movie S4, tricolor imaging of HEK 293T cells for simultaneous  $\text{Ca}^{2+}$ , mitochondrial voltage, and plasma membrane voltage monitoring (AVI)

## ■ AUTHOR INFORMATION

### Corresponding Author

Evan W. Miller – Department of Chemistry, Department of Molecular & Cell Biology, and Helen Wills Neuroscience Institute, University of California, Berkeley, California 94720, United States; [orcid.org/0000-0002-6556-7679](https://orcid.org/0000-0002-6556-7679); Email: [evanwmiller@berkeley.edu](mailto:evanwmiller@berkeley.edu)

### Authors

Pavel E. Z. Klier – Department of Chemistry, University of California, Berkeley, California 94720, United States

Julia G. Martin – Department of Chemistry, University of California, Berkeley, California 94720, United States

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/jacs.0c13110>

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

Research in the Miller lab is supported by the NIH (R35GM119855). E.W.M. acknowledges support from the Camille Dreyfus Teacher Scholar Awards program. P.E.Z.K. and J.G.M. were supported in part by a training grant from the NIH (T32GM066698). Confocal imaging experiments were conducted at the CRL Molecular Imaging Center, supported by the Gordon and Betty Moore Foundation. We would like to thank Holly Aaron and Feather Ives for training and assistance with microscopy. We thank Hasan Celik and the staff of the College of Chemistry NMR facility for their assistance. Instruments in CoC-NMR are supported in part by NIH S10OD024998.

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